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13. ABSTRACT *(Maximum 200 Words)*

Transforming Growth Factor-ßs (TGFß)are polypeptides that are constitutively secreted and activated by many breast carcinomas. They contribute to the tumor's ability to invade and metastasize, to induce angiogenesis and to escape from immune destruction. These circumstances raise the question whether blocking the effects of tumor-derived TGFß on normal tissue (stroma, bloodvessels and immune cells) could be developed as a novel approach to the treatment of breast cancer. We propose to block TGFß action by developing small molecules that inhibit the type I TGFß receptor kinase, which is the key molecule that initiates and mediates TGFß signaling. We plan to develop a cell free ELISA-type assay for high-throughput screening for selective inhibitors of TßR-I kinase activity by using an antibody that specifically detects the phosphorylated form of its substrate, Smad2. Combinatorial libraries of small molecules will then be screened for potent and highly selective for the TßR-I kinase. These will then be tested against normal cells *in vitro* using a number of different assays for TGFß's biological effects. Promising compounds will then be tested for their antitumor activity against highly metastatic, - angiogenic and immunogenic varieties of transplantable breast cancers in mice.

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INTRODUCTION:

Transforming Growth Factor-ßs (TGFß)are polypeptides that are constitutively secreted and activated by many breas carcinomas. They contribute to the tumor's ability to invade and metastasize, to induce angiogenesis and to escape from immune destruction. These circumstances raise the question whether blocking the effects of tumor-derived TGFß on normal tissue (stroma, bloodvessels and immune cells) could be developed as a novel approach to the treatment of breast cancer. We propose to block TGFß action by developing small molecules that inhibit the type I TGFß receptor kinase, which is the key molecule that initiates and mediates TGFß signaling. We planned to develop cell free ELISA-type assay for high-throughput screening for selective inhibitors of TßR-I kinase activity by using a antibody that specifically detects the phosphorylated form of its substrate, Smad2. Combinatorial libraries of small molecules would then be screened for potent and highly selective for the TßR-I kinase. These will then be tested against normal cells *in vitro* using a number of different assays for TGFß's biological effects. Promising compounds will then be tested for their antitumor activity against highly metastatic, -angiogenic and immunogenic varieties of transplantable breast cancers in mice.

BODY:

Tasks 1 & 2. Development of high-throughput assay for specific inhibitors of TßR-I kinase and Screening of combinatorial libraries for potent and specific TßR-I kinase inhibitors

At this point last year, we had conducted a number of feasibility studies that clearly indicated that the aims of this proposal were realistic and could reasonably be expected to be achieved within this funding cycle: We had been able to generate sufficient amounts of both the target enzyme (TßR-I) and its specific substrate (Smad2), and had shown that we could reliably detect kinase activity using both isotopic and non-isotopic methods. A key reagent in this regard is a rabbit antiserum that we had developed, which is highly specific for the phosphorylated forms of the physiological substrate of TßR-I, Smad2.

Although we had intended to develop a high-throughput screening assay for small molecular selective inhibitors of the TßR-I receptor kinase, and screen compound libraries ourselves, we became aware of parallel efforts by a biotech company, Scios, Inc. We were fortunate enough to be able to obtain from Scios, Inc. several small molecules that they had identified in a high-throughput *in vitro* screen as having TßR-I kinase inhibitory activity with varying degrees of potency and selectivity. This fortuitous development has allowed us to pursue Task 3 without delay.

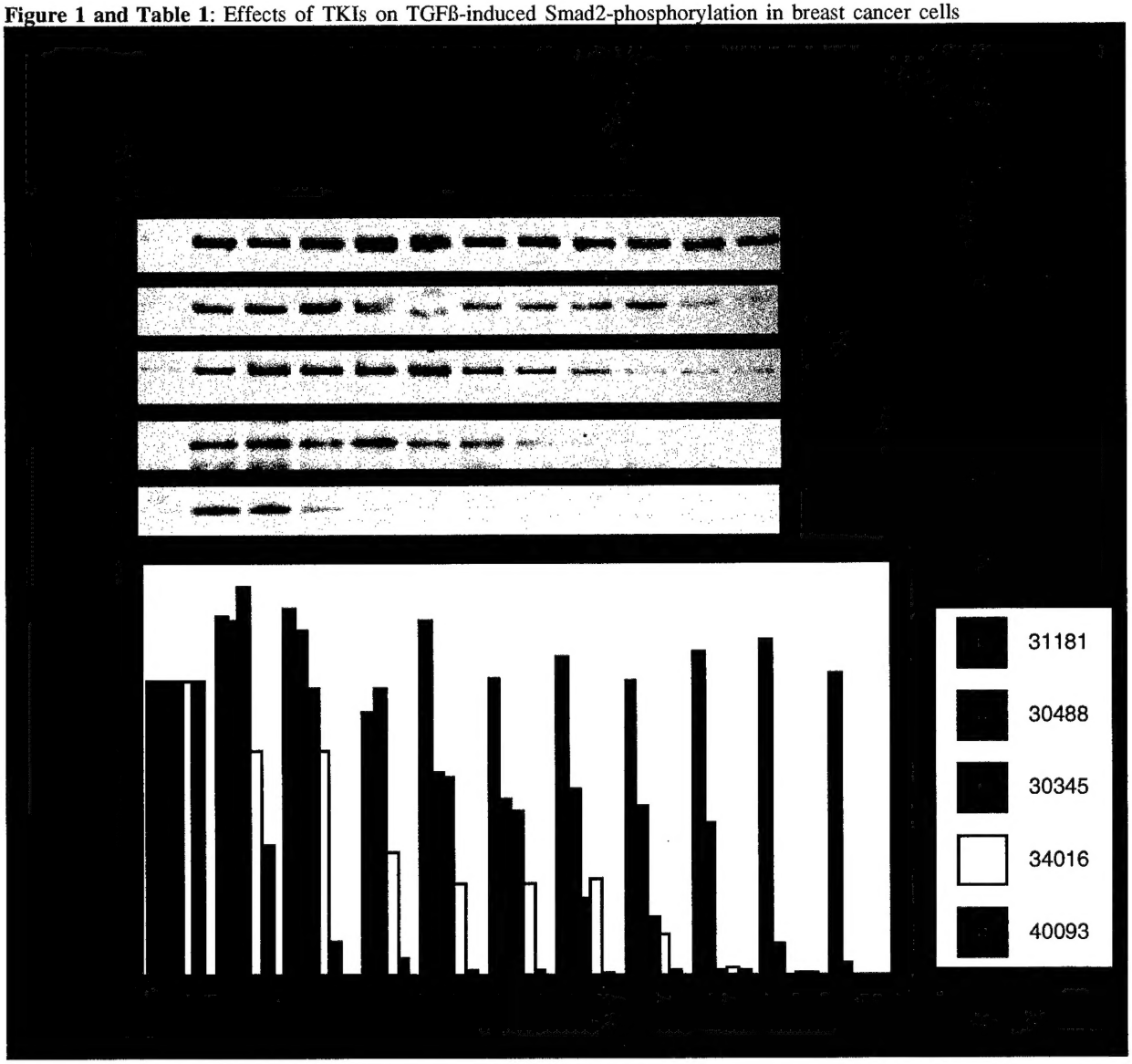
Task 3. Pre-clinical testing of TßR-I kinase inhibitors *in vitro*

a. Effects of TKIs on TGFß-induced Smad2 phosphorylation (Months 18-30)

Effects of TßR-I kinase inhibitors on TGFß signal transduction using cell-based assays. Because many compounds that inhibit a particular biochemical target in cell free systems fail to achieve the same effects in whole cells, we have examined the effects of the Scios lead compounds on TGFß-mediated effects using several different cell-based assays.

***Effects of TRKIs on Smad2 phosphorylation in whole cells*:** Because it is our goal to develop highly specific inhibitors of TßR-I kinase activity for clinical use, it would be highly advantageous to be able to detect its specific phosphorylated substrate *in vitro* and *in vivo*. For this reason, we have produced a polyclonal rabbit antibody that specifically recognizes Smad2 only in its phosphorylated state (see previous annual report). **Figure 1** shows that TßR-I kinase inhibitors block Smad2 phosphorylation in human breast cancer cells. Briefly, semi-confluent human breast carcinoma cells, MDA-MB-435, were treated with varying concentrations of TRKIs followed, 15 minutes later, by the addition of TGFß (100 pM). Following a 2h incubation at 37°C, cells were lysed and the extracts immunoblotted with the anti-Smad2P antibody. The antibody detects a band of approximately 58 kDa representing Smad2P but does not react with unphosphorylated Smad2 in untreated cells. NPC-30488, -30345, -34016 and –40093 represent progressively more potent inhibitors of TGFß-induced Smad2 activation. The control drug, NPC-31181 had no effect. **Table 1** summarizes the estimated IC_{50}'s. NPC-40093 is currently the most potent compound, with an IC_{50} of 20 nM.

Figure 1 and Table 1: Effects of TKIs on TGFß-induced Smad2-phosphorylation in breast cancer cells



	MDA-MB-435	ZR-75-1
31181	>10	>10
30488	0.30	0.55
30345	0.25	0.20
34016	0.06	ND
40093	0.02	ND

b. Effects of TKIs on reporter gene constructs (Months 18-30)

Effects of TRKIs on TGFß-regulated genes: In order to determine the effects of the Scios TRKIs on TGFß-regulated gene expression, we carried out transient transfection assays using a number of different reporter gene assays in Mv1Lu mink lung epithelial cells, which are exquisitely sensitive to TGFß (**Figure 2**). Three different firefly luciferase reporter gene constructs were used: pSBE4 in which 4 tandem repeats of a Smad4-specific DNA binding element (SBE) drive luciferase [Zawel, 1998 #1403]; p3TP-Lux, which contains TGFß-response elements from the collagenase and PAI-1 gene promoters as well as 3 tetradecanoyl phorbol acetate-response elements [Wrana, 1992 #97]; and p15P751-luc (Dr. X.F. Wang, Duke University), which contains the INK4B gene promoter. NPC-30345 was the most potent inhibitor of TGFß-dependent 3TP-Lux activation, with an IC_{50} of approximately 0.6 µM. NPC-30488 was somewhat less active, with an IC_{50} of ~1.25 µM, while the control drug, NPC-31181, had no effect. Qualitatively similar results were obtained with the pSBE4- and INK4B promoter constructs. Of note, the INK4B promoter was relatively less affected, perhaps because of a confounding effect of NPC-30345 on mitogenic signaling pathway kinases.

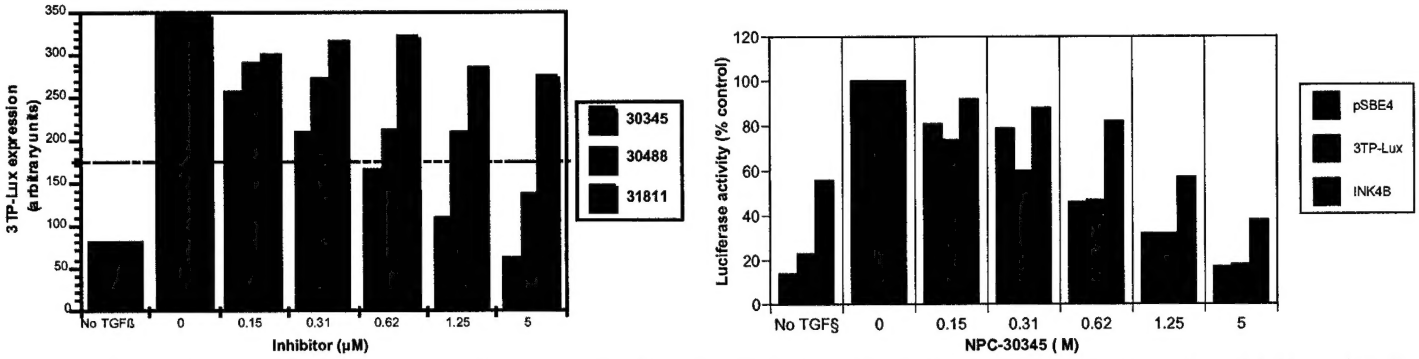


Figure 2. Effects of TßR-kinase inhibitors on TGFß-mediated transcriptional responses. Top: Mv1Lu mink lung epithelial cells were transfected using Lipofectamine 2000 with 3TP-Lux. Four hours later, cells were treated with TRKIs or vehicle only, followed by the addition of TGFß (100 pM) 15 min. later. Luciferase activities in cell extracts were measured 24 hours later. Results were normalized for *Renilla* luciferase activity to correct for differences in transfection efficiency between experiments. Both lead compounds (30345 and 30488) inhibited 3TP-Lux induction by TGFß, while p38_-specific KI 31181 had no effect. Bottom: NPC-30345 inhibits all three TGFß-regulated reporter gene constructs.

c. Effects of TKIs on cell proliferation (Months 18-30)

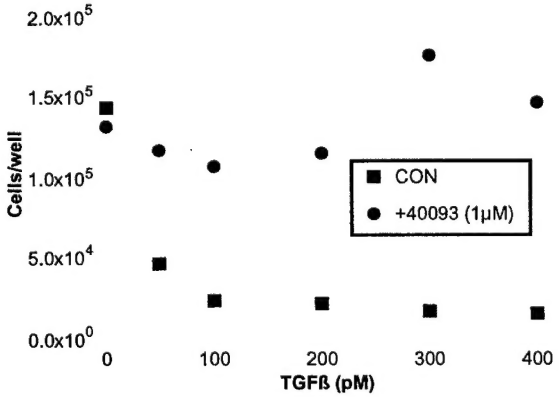


Figure 3: NPC-40093 blocks TGFß-mediated growth inhibition of NMuMG cells. Mv1Lu cells were plated at $2x10^{-4}$ cells/well in 24-well plates. After 4h, NPC-40093 was added at the indicated concentrations, followed by TGFß1 (100 pM) 15 min later. Cells were detached and counted after 72 h. TGFß inhibited cell growth by 90% with an IC_{50} of <50pM. Treatment with NPC-40093 (1 µM) completely blocked TGFß-mediated growth inhibition.

Effects of TRKIs on cell proliferation: TGFß is the most potent known inhibitor of cell cycle progression of non-malignant epithelial cells. To determine whether or not the TRKIs were able to inhibit TGFß-mediated growth inhibition, we treated NMuMG (normal mouse mammary gland) cells that are extremely sensitive to TGFß with varying concentrations of TRKIs (**Figure 3**). Treatment with NPC-40093 completely reversed TGFß-mediated growth inhibition in a dose-dependent manner.

'd. Effects of TKIs on extracellular matrix-associated proteins (Months 18-30)

Besides causing growth arrest, TGFß induces the production of plasminogen activator inhibitor type 1 and collagenases, as well as ECM proteins, such as fibronectin (FN). Both 30345 and 30488 inhibited PAI-1 synthesis by human cardiac fibroblasts in a dose-dependent manner (**Figure 4**).

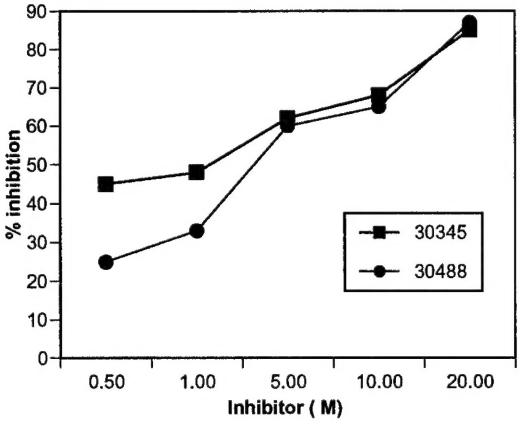


Figure 4: Inhibition of TGFß-induced PAI-1 synthesis. Primary human cardiac fibroblasts were treated with TGFß1 (400 pM) in the presence or absence of TßR-I kinase inhibitors, 30345 and –30488. After 48h, conditioned medium was collected and the PAI-1 concentration determined using an ELISA kit (American Diagnostica, Greenwich, CT). 30345 inhibited TGFß-induced PAI-1 secretion with an IC_{50} between 0.5-1 µM.

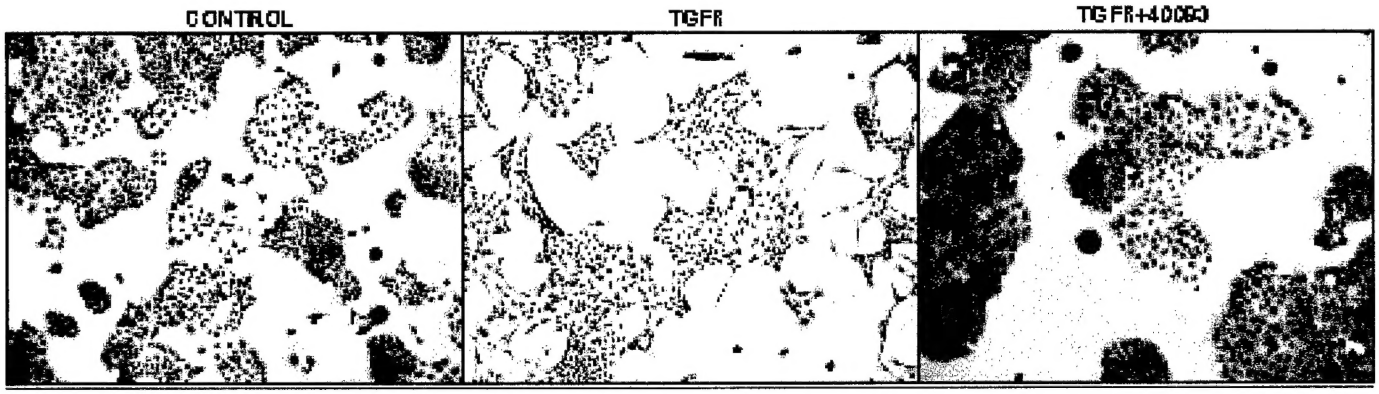


Figure 5: Inhibition of TGFß-induced epithelial-to-mesenchymal transition (EMT). NMuMG normal mouse mammary gland cells were treated with TGFß1 (100 pM) in the presence or absence of NPC-40093. After 36h, cells were fixed and stained with hematoxyllin-eosin. TGFß induced EMT, as manifested by the cells assuming a spindle-shaped morphology and scattering. In the presence of NPC-40093 (1 µM), induction of EMT by TGFß was completely blocked.

Effect of TRKIs on epithelial-to-mesenchymal transition (EMT): In certain epithelial cell types, such as keratinocytes and mammary epithelial cells, treatment with TGFß induces a process of transdifferentiation characterized by scattering, increased motility and the acquisition of a fibroblastoid (spindle-shaped) morphology. As shown in **Figure 5**, TGFß induces EMT in NMuMG mouse mammary epithelial cells. This effect can be completely prevented in the presence of the TßR-I kinase inhibitor, NPC-40093, indicating that this process is mediated by the TßR-I receptor kinase.

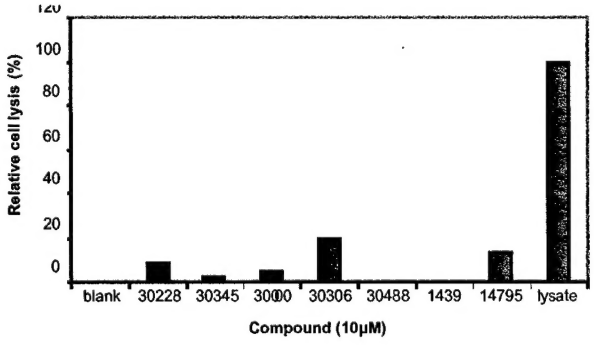


Figure 6: Absence of cytotoxicity in human cardiac fibroblasts treated with TßR-I antagonists. Cells were treated with various kinase inhibitors (10µM) for 48h. Drug cytotoxicity is reflected by release of lactate dehydrogenase (LDH) upon cell lysis. LDH in culture supernatants was measured with a 30-minute coupled enzymatic assay, which results in conversion of a tetrazolium salt (INT) into a red formazan product that can be read at 490 nm using a 96 well plate reader. The amount of color formed is proportional to the number of cells lysed.

Effect of TRKIs on cell viability: Finally, we determined whether or the TßR-I kinase inhibitors derived from the HTS possessed any direct cytotoxic effects on human cardiac fibroblasts using the LDH-release assay. As shown in **Figure 6**, neither NPC-30345 nor –NPC-30488 had any direct cytotoxic effects at 10 μM.

The qualitatively and quantitatively similar effects of the lead TßR-I antagonists on TGFß-dependent effects on gene expression, TßR-driven Smad2 phosphorylation, as well as global effects on cell growth and synthesis of ECM-related proteins are quite remarkable. These findings clearly indicate not only that these compounds are capable of potently inhibiting TßR kinase activity, but also that they cross the plasma membrane quite efficiently and are able to effectively block TGFß signaling in whole cells.

Task 4. Pre-clinical testing of TßR-I kinase inhibitors *in vivo*

a. Effects of TKIs on growth of highly immunogenic transplantable mammary carcinoma lines in mice - Approximately 45 mice per compound (Months 24-36)

b. Effects of TKIs on growth of highly metastatic breast cancer - Approximately 45 mice per compound (Months 24-36)

c. Effects of TKIs on growth of highly angiogenic breast cancer - Approximately 45 mice per compound (Months 24-36)

KEY RESEARCH ACCOMPLISHMENTS:

- Development of selective and potent small molecular chemical inhibitors of TGFß type I receptor kinase (TKIs)
- TKIs inhibit TGFß-induced Smad2-phosphorylation in whole cells without causing cell toxicity
- TKIs block TGFß-mediated regulation of target genes
- TKIs block TGFß-induced inhibition of cell growth
- TKIs block TGFß-mediated induction of extracellular matrix associated proteins
- TKIs block TGFß-induced epithelial-to-mesenchymal transition of mouse mammary epithelial cells

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Rongrong Ge, David Liu, Alison Joly, Sundeep Dugar, Jit Chakravarty, Margaret Henson, Glenn McEnroe, George Schreiner and **Michael Reiss**. Selective Inhibition of Transforming Growth Factor-ß Signaling Blocks Invasiveness of Human Breast Carcinoma Cells In Vitro. Proc. Am. Assoc. Cancer Res. 43:958. 2002.

Reiss, M. Transforming Growth Factor-[beta] and cancer. In: "Cytokines in Liver Injury and Repair – Falk Symposium 125" Gressner et al. (eds). Kluwer Academic Publishers, Amsterdam. 2002. (In Press)

CONCLUSIONS: Summarize the results to include the Importance and/or implications of the completed research an when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

Late stages of breast cancer development and progression are associated with activation of TGFß in the tumor microenvironment. This bioactive TGFß is thought to enhance tumor progression by both autocrine effects on the tumor cells themselves and paracrine effects on stromal cells, endothelial cells, and immune cells that promote invasion, angiogenesis and escape from immune surveillance, respectively. The overall aim of this project is to develo small molecular chemical inhibitors of TGFß signaling and test their efficacy against advanced breast cancer in anim models.

Since we began this project little over one year ago, we have made significant progress. Thanks to a fortuitous collaboration with scientists at Scios, Inc., we have obtained access to small molecules that selectively and potently target the TGFß type I receptor kinase *in vitro*. Our studies of these compounds over the past year have established that these TKIs (1) TKIs inhibit TGFß-induced Smad2-phosphorylation in whole cells without causing cell toxicity; (2) TKIs block TGFß-mediated regulation of target genes; (3) TKIs block TGFß-induced inhibition of cell growth;

(4) TKIs block TGFß-mediated induction of extracellular matrix associated proteins; (5) TKIs block TGFß-induced epithelial-to-mesenchymal transition of mouse mammary epithelial cells. Thus, we are poised to embark on a series o studies using animal models to test their anti-cancer effects in vivo, and determine whether their activity targets is primarily mediated by inhibition of metastasis or angiogenesis, or by enhancing anti-tumor immunity.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in Science, Military Medicine, etc.).

Reiss, M. and Barcellos-Hoff, M.H. Transforming Growth Factor-ß in breast cancer-a working hypothesis-Breast Cancer Res. & Treatment. 1997. 45:81-95.

Reiss M., TGF-beta and cancer. Microbes Infect. 1:1327-1347, 1999.